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ORIGINAL ARTICLE

Carbon monoxide inhibits apoptosis during cold storage and protects kidney grafts donated after cardiac death

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Summary

Ischemia/reperfusion (I/R) injury remains as a serious deleterious factor in kidney transplantation (KTx). We hypothesized that carbon monoxide (CO), an endogenous potent cytoprotective molecule, inhibits hypothermia-induced apoptosis of kidney grafts. Using the rat KTx model mimicking the conditions of donation after cardiac death (DCD) as well as nontransplantable human kidney grafts, this study examined effects of CO in preservation solution in improving the quality of marginal kidney grafts. After cardiac cessation, rat kidneys underwent 40 min warm ischemia (WI) and 24 h cold storage (CS) in control UW or UW containing CO (CO-UW). At the end of CS, kidney grafts in control UW markedly increased mitochondrial porin release into the cytosol and resulted in increased cleaved caspase-3 and PARP expression. In contrast, grafts in CO-UW had significantly reduced mitochondrial breakdown and caspase pathway activation. After KTx, recipient survival significantly improved with CO-UW with less $TUNEL^+$ cells and reduced mRNA upregulation for proinflammatory mediators (IL-6, TNF-α, iNOS). Furthermore, when nontransplantable human kidney grafts were stored in CO-UW for 24 h, graft PARP expression, $TUNEL^+$ cells, and proinflammatory mediators were less than those in control UW. CO in UW inhibited hypothermia-induced apoptosis and significantly improved kidney graft function and outcomes of KTx.

Introduction

Ischemia/reperfusion injury (I/R) of the kidney allograft has been considered to be one of the major deleterious factors limiting the success of kidney transplantation (KTx). I/R injury increases the risk of delayed graft function (DGF) and complicates early post-transplant recipient management. I/R injury also causes progressive graft dysfunction with chronic fibroinflammatory changes and impacts on long-term graft survival [1–3]. In the current organ shortage era, the donor pool has been expanded with an increasing use of kidneys from expanded criteria donors (ECD) and donation after cardiac death (DCD). As the marginal kidney grafts may suffer from significant I/R injury and have a high incidence of DGF, strategies to improve the quality of these grafts may have a significant impact on short- and long-term outcomes after KTx.

In the conventional view, carbon monoxide (CO) is a poison because of its ability to interfere with oxygen delivery in high concentrations. However, mammalian cells endogenously generate low concentrations of CO primarily via the catalysis of heme by hemeoxygenases (HO), and the constitutive HO and endogenous generation of CO have been shown to be an essential mechanism to

maintain the integrity of the physiological function of organs [4,5]. In fact, exogenously delivered CO at low concentrations has been shown to provide cytoprotection in numerous experimental injury models [6]. In the experimental organ transplantation models, we have shown that inhaled or *ex vivo* delivered CO prevents I/R injury in various organ grafts [7]. In particular, CO effectively inhibited renal I/R injury after KTx in rodents [8–10] and large animals [11,12].

In this study, we expanded our research to explore the capability of CO in preventing I/R injury in kidney grafts obtained from DCD or ECD donors. We hypothesized that CO in UW preservation solution could inhibit the progression of injury in kidney grafts during the cold storage (CS) period. Using the rodent model of KTx with DCD donors, as well as human ECD kidney grafts that were deemed nontransplantable, this study demonstrates that *ex vivo* application of CO in UW CS solution inhibits apoptosis pathway activation during CS, reduces renal I/R injury and improves early post-transplant kidney graft function and recipient survival.

Materials and methods

Reagents

Rabbit polyclonal antibodies for cleaved caspase-3 (Cell Signaling Technology, Danvers, MA, USA), poly (ADP-ribose) polymerase (PARP) (Cell Signaling Technology), porin (Calbiochem, Gibbstown, NJ, USA), and β-actin (Sigma-Aldrich, St Louis, MO, USA), and mouse monoclonal antibody for $gp91^{phox}$ (BD Biosciences, San Jose, CA, USA) were used. For secondary antibodies, goat anti-rabbit or -mouse antibodies (Pierce Chemical, Rockford, IL, USA) were used.

CO supplementation to UW solution

For each transplant experiment, 50 ml UW solution was vigorously bubbled for 5 min at 4 °C with compressed 5% CO gas mixed in air (Valley National Gases, Pittsburgh, PA, USA) (CO-UW). CO solubility in CO-UW was 40.6 ± 1.6 μmol/l [13].

Animals and KTx procedures with warm and cold ischemia

Inbred male Lewis ($RT.1^{l}$) rats weighing 200–250 grams (Harlan Sprague Dawley, Inc., Indianapolis, IN, USA) were maintained with a standard diet and water supplied *ad libitum*. In the DCD KTx model under isoflurane anesthesia, the left kidney, renal vessels and ureter were isolated via a midline incision. After intravenous heparinization (300 U), cardiac arrest was induced by opening the chest. The cessation of the heart beat was observed within 3–5 min, and donor animals were kept for 40 min on a 37 degree pad [14,15]. The left kidney then was removed with the left renal artery and a short aortic segment, and the left renal vein with a patch of the vena cava. The excised graft was flushed with and preserved in control UW or CO-UW at 4 °C for 24 h. The kidney grafts were orthotopically transplanted into syngeneic recipients by end-to-side anastomoses between graft aorta and recipient infrarenal abdominal aorta, and between graft renal vein and recipient infrarenal vena cava with end-to-end ureteral anastomosis [8,9]. Both native kidneys were removed at the time of KTx. All procedures were performed according to the guidelines of the National Research Council's Guide for the Humane Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh.

Kidney graft samples were obtained at the end of 40 min warm ischemia (WI) and at the end of 24 h CS, as well as at 1, 3, 6, and 24 h after KTx. Samples were fixed in 10% buffered formalin, or snap-frozen and stored at −80 °C. In additional sets of experiments, recipient animals were followed for 10 days to assess renal graft function and animal survival.

Renal functions

Serum creatinine levels were measured using a Beckman autoanalyzer (Beckman Instruments, Fullerton, CA, USA).

Histopathology and immunohistochemistry

Kidney graft tissues were fixed in 10% formalin, embedded in paraffin, sectioned into 6 μm thickness, and stained with hematoxylin and eosin (H&E). Paraffin-fixed sections were also stained with anti-cleaved caspase-3 antibody and apoptotic cells were detected by the TUNEL assay. Positively stained cells were counted in a blind fashion in 10 random cortical high-power fields (×400) per section. Masson's trichrome stain was performed, and positively stained fibrotic area in five random low-power fields (×100) was morphometrically quantified using NIH image software.

RNA extraction and SYBR Green real time RT-PCR

RNA was isolated from the kidney grafts and 3 μg of RNA from each sample was used to generate first-strand cDNA [8]. PCR reaction mixture was prepared using SYBR Green PCR Master Mix (PE Applied Biosystems, Foster City, CA, USA) using the previously published primers [8], and PCR was performed with an ABI PRISM 7000 Sequence Detection System (PE Applied Biosystems). The

expression of each target gene was normalized to housekeeping GAPDH mRNA content and calculated relative to normal kidney control using comparative Ct methods [16,17].

Western blot analysis

Western blot analysis was performed using 50 μg of cytoplasmic, cytosolic, or mitochondrial protein from graft tissues as previously described [18]. After the blocking with nonfat dry milk, membranes were incubated overnight with primary antibodies, followed with secondary antibodies. Membranes were developed with the SuperSignal® West Femto Maximum Sensitivity Substrate detection systems (Pierce Chemical) and exposed to film. The band intensities were measured by NIH Image Analysis software.

Measurement of adenylates and their metabolites

Kidney samples were immediately frozen in liquid nitrogen until the extraction procedure. The frozen tissue was homogenized in 1.0 ml of ice-cold 6% perchloric acid containing 0.77 mM EDTA and centrifuged for 10 min at 10 000 g at 4 °C. The concentration of ATP, ADP, and AMP, hypoxanthine, xanthine, and inosine were measured by HPLC at 254 nm (Waters, Inc., Milford, MA, USA) and calculated from a standard curve constructed with standard powders (>99% pure, Sigma-Aldrich) [19].

Tissue CO Contents

The tissue CO content was measured using homogenized graft samples and TRI lyzer (Taiyo, Osaka, Japan) with the method previously described [20]. The results were expressed as picomoles of CO per milligram of tissue.

Human kidney grafts

Human kidney grafts that were deemed nontransplantable were obtained from our local OPO and used for control and CO-UW static storage experiments, which was approved by the Committee for Oversight of Research Involving the Dead (CORID) of the University of Pittsburgh and the Center for Organ Recovery & Education (CORE). As soon as the nontransplantable kidney graft became available for research, a baseline sample was obtained. A catheter was inserted into the upper renal arterial branch, and 100 ml CO-UW (10%) was perfused, while the lower part of the kidney was perfused with 100 ml control UW. The kidney graft was split into upper and lower portions, and stored for 24 h in CO-UW and control UW solution respectively. After 24 h cold static storage, kidney graft samples were obtained for CO contents, histopathology, Western blot, and real time RT-PCR. In addition, explants were obtained by precisely cutting kidney grafts into 2.0 × 5.0 × 5.0 mm cubes using straight adjustable tissue cutting channel TM-S-9 (Braintree Scientific, Inc. Braintree, MA, USA), and incubated in 12-well plates with 1.0 ml of Dulbecco's Modified Eagle's Medium, supplemented with 4.5 g/l glucose, 2 mM, L-glutamine, 5% FBS, 0.4 IE/ml insulin, 20 mM HEPES, and 50 μg/ml gentamycin. Plates were cultured for 3, 6, and 12 h at 37 °C under gentle shaking in a conventional 5% CO_2 incubator [21]. In this human kidney study, we used one kidney graft for both control UW and CO-UW to have the same baseline values, as histopathological findings were different between two kidneys from the same donor. Proper perfusion with control UW and CO-UW was confirmed by measuring tissue CO levels.

Statistical analysis

Data are represented as the mean ± SD. Comparisons between groups at different time points were performed using Student's *t*-test or ANOVA. Graft survivals were plotted with the Kaplan–Meier method, and the difference among groups was analyzed using the log-rank test. Differences were considered significant at a P-value < 0.05.

Results

CO inhibits apoptosis in kidney grafts during CS

Mitochondria contain many pro-apoptotic proteins (e.g. AIF, cytochrome c), and stress/injury has been shown to induce pore formation in the mitochondrial membrane, release mitochondrial proteins to the cytosol, and activate apoptosis pathways [22,23]. To determine whether CS-induced mitochondrial protein release in kidney grafts, the cytosol protein fraction was obtained from kidney grafts and expression of porin was examined. Although porin expression was not evident in normal kidney cytosol samples, 40 min WI or 24 h CS in control UW solution alone resulted in increases of porin in cytosol protein fractions. WI followed by CS in control UW strikingly increased porin expressions. In contrast, porin release was significantly inhibited in WI followed by CS in CO-UW (Fig. 1). Subsequent activation of apoptosis pathways was analyzed by cleaved caspase-3 and PARP expression in kidney graft cytoplasmic proteins. Marginal expression of cleaved caspase 3 in kidney grafts after WI or CS was significantly augmented after WI plus CS in control UW, while CO-UW showed reduced expression. Likewise, cleaved PARP expression at the end of CS was significantly inhibited with CO-UW compared to control UW (Fig. 1a and b).

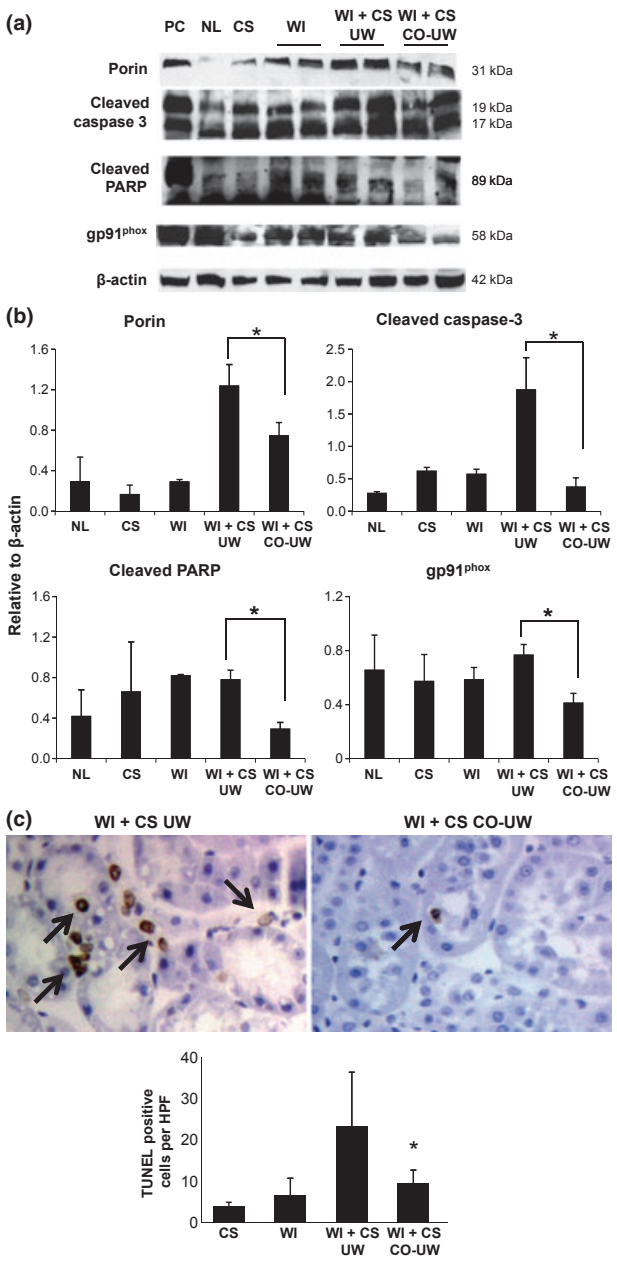

Figure 1 CO in UW inhibits mitochondria breakdown and apoptosis induction in kidney grafts during cold storage. (a) Cytoplasmic, cytosolic and mitochondrial protein fractions from kidney grafts were analyzed in Western blot for mitochomdrial porin (cytosolic fractions), cleaved caspase 3, cleaved PARP and gp91^{phox} (cytoplasmic fractions). Kidney grafts were obtained after 40 min warm ischemia (WI), 24 h cold storage in control UW (CS), or 40 min WI followed by 24 h CS in control UW (WI + CS UW) or in CO-UW (WI + CS CO-UW). Representative experiment is shown. (b) Band intensities were quantified and expressed as ratios to those of β-actin. (c) TUNEL analysis of kidney grafts. Positively stained cells (arrows, brown) in kidney grafts were counted in 10 randomly selected high power fields (×400) per each section. *$P < 0.05$ using $n = 3$–4 samples for each group. PC, positive control; NL, normal rat kidney.

NADPH oxidase plays a crucial role in generating superoxide, and gp91^{phox}, a membrane-bound subunit of NADPH oxidase, has been shown to be an oxidative stress marker [24]. Western blot analysis revealed that during WI and/or CS, gp91^{phox} expression was not altered in kidney grafts. However, when grafts were kept in CO-UW, gp91^{phox} expression was significantly reduced when compared to that in control UW (Fig. 1a and b).

Numerous TUNEL-positive cells were found in kidney grafts before transplantation, when kidney grafts underwent 40 min WI and were preserved in control UW for 24 h. Grafts stored in CO-UW had significantly less apoptotic cells when compared to those stored in control UW (Fig. 1c). The majority of TUNEL^{+} cells were tubular epithelial cells (arrows, Fig. 1c).

Energy status and adenylate (ATP, ADP, and AMP) levels

Kidney samples at the end of CS were also analyzed with HPLC for adenylates and their metabolite levels. Compared to naïve kidneys, ATP, ADP, and AMP levels were significantly reduced in grafts after WI and 24 h CS in control UW. CO-UW did not affect kidney graft energy status, and there were no differences between control UW and CO-UW (Fig. 2a). Likewise, WI plus CS resulted in accumulations of metabolites, and both control UW and CO-UW resulted in increased hypoxantine, xantine, inosine, and adenosine tissue levels before KTx (Fig. 2b).

These results suggest that the combination of WI and CS induces significant kidney damage as evident by marked releases of mitochondrial porins into the cytosol and increased expression of cleaved caspase-3 and PARP. CS of grafts in CO-UW effectively reduced mitochondrial breakdown and caspase pathway activation.

CO in UW improves kidney graft function after KTx

To evaluate the protective effects of CO-UW during CS period on post-transplant renal function, rat kidney grafts underwent WI and CS in control UW or CO-UW and then were transplanted into syngeneic recipients. Serum creatinine levels promptly increased in control UW group after KTx and reached 7.9 ± 2.1 mg/dl on day 3 (Fig. 3a). In contrast, CO-UW group showed significantly lower peak creatinine levels (4.6 ± 2.1 mg/dl at day 2). Accordingly, the majority of animals in control UW group died within 7 days, and only two of six survived for 10 days. In contrast, all six animals survived for 10 days in CO-UW group (Fig. 3b). H&E stain and Masson's trichrome stain of the kidney graft samples obtained from

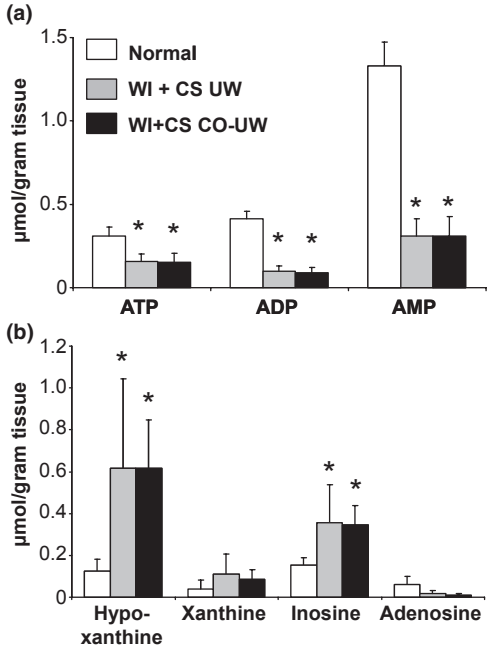

Figure 2 Adenylates and their metabolites in kidney grafts at the end of warm ischemia (WI) and CS. Kidney graft samples were obtained after 40 min WI followed by 24 h CS in control UW (WI + CS UW) or in CO-UW (WI + CS CO-UW) and analyzed with HPLC for (a) adenylates and (b) metabolites. $*P < 0.05$ vs. normal kidney using $n = 3$–4 samples for each group.

surviving animals at 10 days after transplantation revealed that interstitial infiltration and tubular loss were more prominent in control UW than in CO-UW group and that interstitial fibrosis was significantly less in CO-UW than in UW group (25.1 ± 2.4 vs. 36.5 ± 4.1%, $P < 0.005$ (Fig. 3c).

CO in UW reduces apoptosis after KTx

TUNEL stain of kidney grafts after KTx showed numerous $TUNEL^+$ tubular epithelial cells; however, the numbers of $TUNEL^+$ cells at 3 h after KTx were significantly lower in CO-UW group (Fig. 4a).

CO in UW inhibits proinflammatory responses in ECD kidney grafts

Storage of kidney grafts with WI and CS in CO-UW resulted in a significant reduction of mRNA upregulation for proinflammatory cytokines. IL-1β, IL-6, IL-8, TNF-α, IFN-γ, and iNOS mRNA levels peaked at 3 h after KTx in control UW group. CO-UW group showed significantly lower mRNA levels for IL-1β, IL-6, IFN-γ, and iNOS (Fig. 4b).

Human kidneys exposed to CO-UW show less apoptosis pathway activation

To further evaluate if storage of human kidney grafts in CO-UW can inhibit apoptosis during CS, we stored five nontransplantable kidney grafts in control UW or CO-UW solution for 24 h. Donor ages were 68 ± 27 years old with serum creatinine levels of 1.6 ± 1.3 mg/dl. The cause of death was CVA, and graft biopsy showed 13.6 ± 7.6% glomerulosclerosis with moderate arteriosclerosis and interstitial fibrosis. The human kidney grafts became available 13.5 ± 3.9 h after graft retrieval and static storage. Tissue CO contents increased after 24 h storage in CO-UW to 2.66 ± 1.37 pmol/mg, while those with control UW were 0.81 ± 0.18 pmol/mg (Fig. 5a). Kidney grafts showed some levels of cleaved caspase-3 and PARP expression when they became available for research. After an additional storage for 24 h, kidney portions stored in control UW showed a 20% increment of cleaved caspase-3 expression compared to pre-treated samples (Fig. 5b). CS in CO-UW resulted in slightly less cleaved caspase-3 expression. PARP expression was reduced in 4% in grafts preserved in UW solution and 14% reduction in those in CO-UW (Fig. 5b).

After explant culture for 3–12 h, there were significantly fewer TUNEL-positive cells when kidneys were preserved in CO-UW compared to control UW (Fig. 5c). In addition, peak mRNA levels for TNF-α and IL-6 were lower with CO-UW than with control UW after explant culture for 3 and 6 h respectively (Fig. 5d).

Discussion

Kidney transplantation has become an established therapeutic choice providing improved longevity and quality of life for patients with end-stage renal disease. Unfortunately, however, there is a shortage in the supply of kidneys available for transplantation, and the waiting list and wait-list times have been growing. The condition of the patients waiting for a kidney transplant can deteriorate, resulting in an increased mortality while waiting for a transplant [25,26]. Although the serious discrepancy between organ supply and demand is recognized, many recovered kidneys are still discarded because of the higher risks of graft loss with marginal donor kidneys [27,28]. In an effort to increase the donor pool, the use of kidneys from ECD/DCD has become common. Although these marginal kidneys are shown to be inferior to standard criteria donor kidneys, they still provide a survival advantage over maintenance dialysis [29]. Therapeutic strategies that could suppress the deterioration of marginal kidney grafts during cold preservation before transplantation

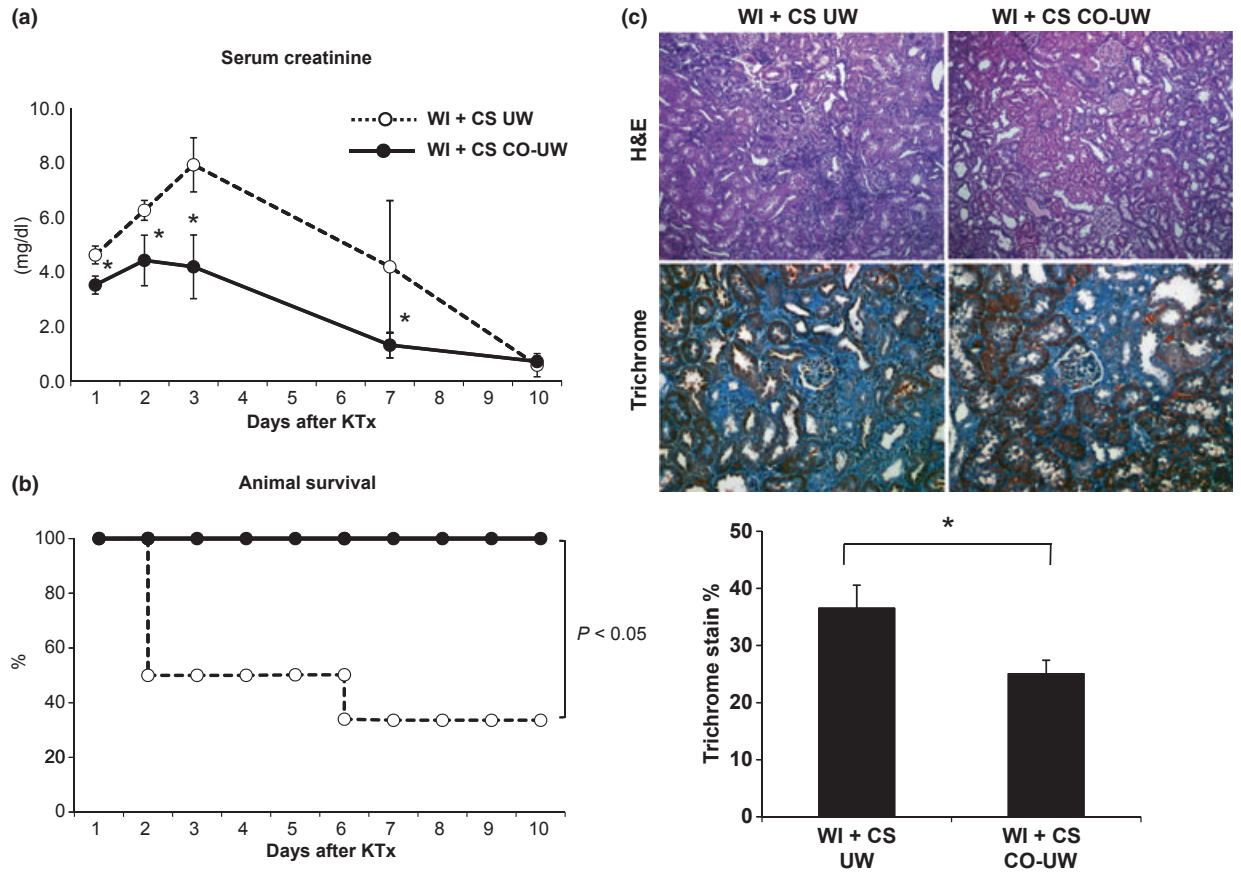

Figure 3 CO in UW improves renal graft function and survival after KTx. Rat kidney grafts underwent warm ischemia (WI) and 24 h CS in control UW (WI + CS UW, $n = 6$) or CO-UW (WI + CS CO-UW, $n = 6$) and were transplanted into syngenic recipients. Recipients were followed for 10 days to determine (a) serum creatinine levels and (b) survival. *$P < 0.05$ WI + CS UW vs. WI + CS CO-UW. (c) H&E stain (upper) and Masson's trichrome stain (lower) of the kidney graft samples obtained at 10 days after KTx. Original magnification ×100. Trichrome-stained fibrotic area was morphometrically quantified by NIH image software. *$P < 0.005$, WI + CS UW ($n = 2$) vs. WI + CS CO-UW ($n = 6$).

might increase safe use of marginal kidneys and reduce discard rates of kidney grafts.

The current study demonstrates that CO added into UW preservation solution protects marginal kidney grafts against hypothermia/hypoxia-induced graft injury during the CS period. In the rat model of DCD kidney grafts with 40 min WI and 24 h CS, *ex vivo* treatment of kidney grafts with CO in UW preservation solution controlled destructive mitochondrial breakdown and caspase pathway activation during CS and improved graft quality prior to KTx. Beneficial effects of *ex vivo* CO treatment of kidney grafts were evident after KTx with reduced early mRNA upregulation for proinflammatory mediators and less $TUNEL^+$ cells in kidney grafts. More importantly, renal graft function and survival were significantly improved with CO-UW. Furthermore, when the study was extended to human kidney grafts deemed nontransplantable, CO in UW solution was able to inhibit apoptosis progression in human kidneys during CS, suggesting that CO might improve the quality of DCD/ECD kidney grafts and could be used as a strategy to improve outcomes of KTx.

Although graft reperfusion is a crucial event to display the graft injury, the initiation of the I/R injury process takes place at a much earlier stage of the transplant procedure. Among many damaging processes associating with KTx, the hypothermic/hypoxic condition during CS is crucial in initiating the injury process in the grafts. Cold perfusion and preservation of organ grafts are the principal modalities to slow down cell metabolism and decrease oxygen and energy consumption; however, hypothermic condition per se induces losses of ionic homeostasis, membrane integrity, and membrane-associated transporter and enzyme functions, resulting in metabolic, structural, and functional cell injury [30–32]. In various types of cultured cells, including renal tubular epithelial cells, hypothermia has been shown to induce apoptosis by increasing the opening of the permeability transition pores and subsequent mitochondrial swelling, generation

of ROS, and direct DNA damage, as well as an activation of proteasomes and serine proteases [33–36]. Accordingly, the CS procedure significantly affects organ graft viability before graft reperfusion. Previous experimental studies have shown that prolonged cold ischemia increased apoptotic cell death in organ grafts, such as the kidney, liver, and lung [34,37,38]. Furthermore, caspase inhibitors added into cold preservation solution have been shown to inhibit the apoptotic process, reduce I/R injury, and improve graft function [37,38]. Moreover, clinical studies demonstrate that increased frequencies of apoptotic renal tubular epithelial cells in kidney graft biopsies before transplantation are associated with the development of DGF [39,40]. Thus, activation of apoptosis during CS plays a significant role in transplant-induced I/R injury and post-transplant graft function. As I/R injury is a cascade of inter-related events with ongoing augmentation of injury, early therapeutic intervention in the process could have significant effects in ameliorating injury and improving outcomes.

As CO has a strong affinity to transition metals, the major molecular targets of CO most likely are iron, the most abundant element of the body's transition metals. Thus, the mechanisms by which CO inhibits hypothermia-induced apoptosis and I/R injury in this study could involve the binding of CO to iron or other transition metals in kidney grafts during CS. CO has been shown to inhibit both intrinsic and extrinsic apoptotic signaling by inhibiting cytochrome c release, ROS generation, and caspase activation, as well as by inhibiting the activation of Bcl-2-related proteins of Bid and Bax, while promoting Bcl-XL/Bax interaction and Bad phosphorylation in the model of hyperoxia-induced endothelial cell death [41]. Further studies to explore the binding sites of CO and mechanisms of anti-apoptotic function of CO in the ECD kidney model are warranted. Recent studies have shown that pulsatile perfusion of kidney grafts significantly reduces the risk of DGF and improves 1-year survival [42–44], and application of therapeutic agents, such as CO, during the machine perfusion would also be interesting future studies.

The experiment in this study was designed to examine effects of CO on I/R injury without any additional factor influencing the endpoints, such as immunosuppression and alloantigens. However, these factors are unavoidably involved in clinical transplantation and could significantly influence I/R injury and graft outcomes. It has been shown that immunosuppressive drugs, in particular carcineurin inhibitors have beneficial effects during warm and cold I/R injury in extrarenal organs. Particularly, hepatic cold I/R injury in liver grafts can be significantly ameliorated with tacrolimus and cyclosporine [45,46]. In contrast, studies

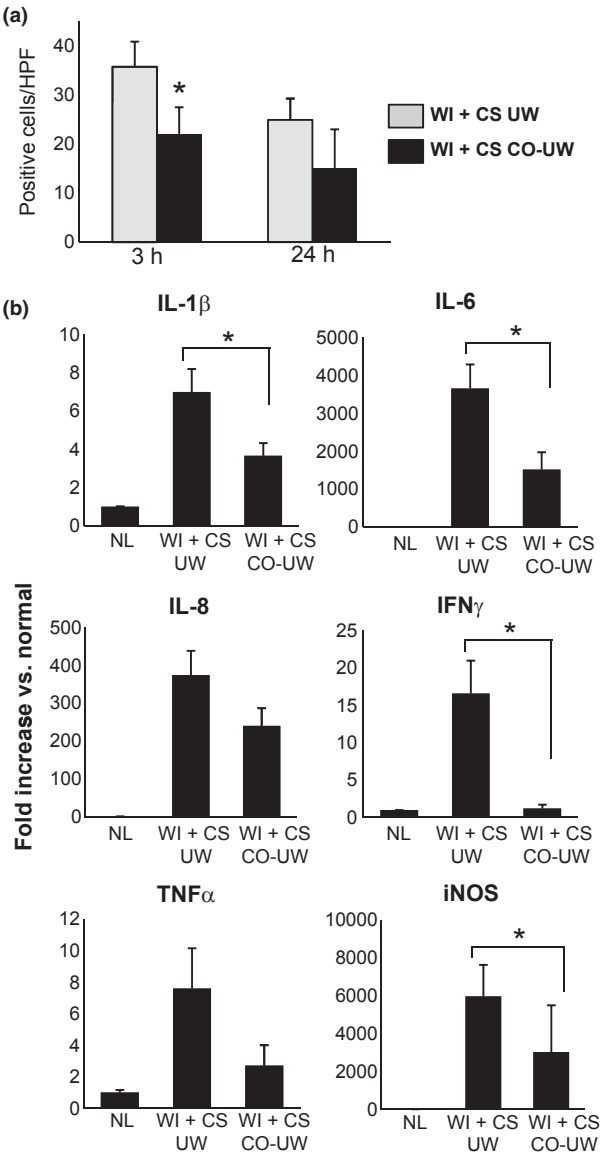

Figure 4 CO in UW reduces renal graft apoptosis and inflammatory responses after KTx. (a) Renal graft apoptosis after KTx. Kidney grafts after warm ischemia (WI) followed by 24 h CS in control UW (WI + CS UW) or in CO-UW (WI + CS CO-UW) were transplanted into syngenic recipients and graft samples were obtained 3 and 24 h after KTx for TUNEL analysis. $TUNEL^+$ cells in kidney grafts were counted in 10 randomly selected fields (×400) per each section. $^*P < 0.05$ vs. control UW $n = 3$–4 samples for each group. (b) Proinflammatory cytokine upregulation after KTx was attenuated in grafts stored in CO-UW: Kidney graft samples were obtained 3 h after KTx and analyzed by RT-PCR for IL-1β, IL-6, IL-8, iNOS, TNF-α and IFN-γ. WI + CS UW: 40 min WI followed by 24 h CS in control UW, WI + CS UW: 40 min WI followed by 24 h CS in CO-UW. $^*P < 0.05$ vs. control using $n = 3$–4 samples for each group. NL, normal rat kidney.

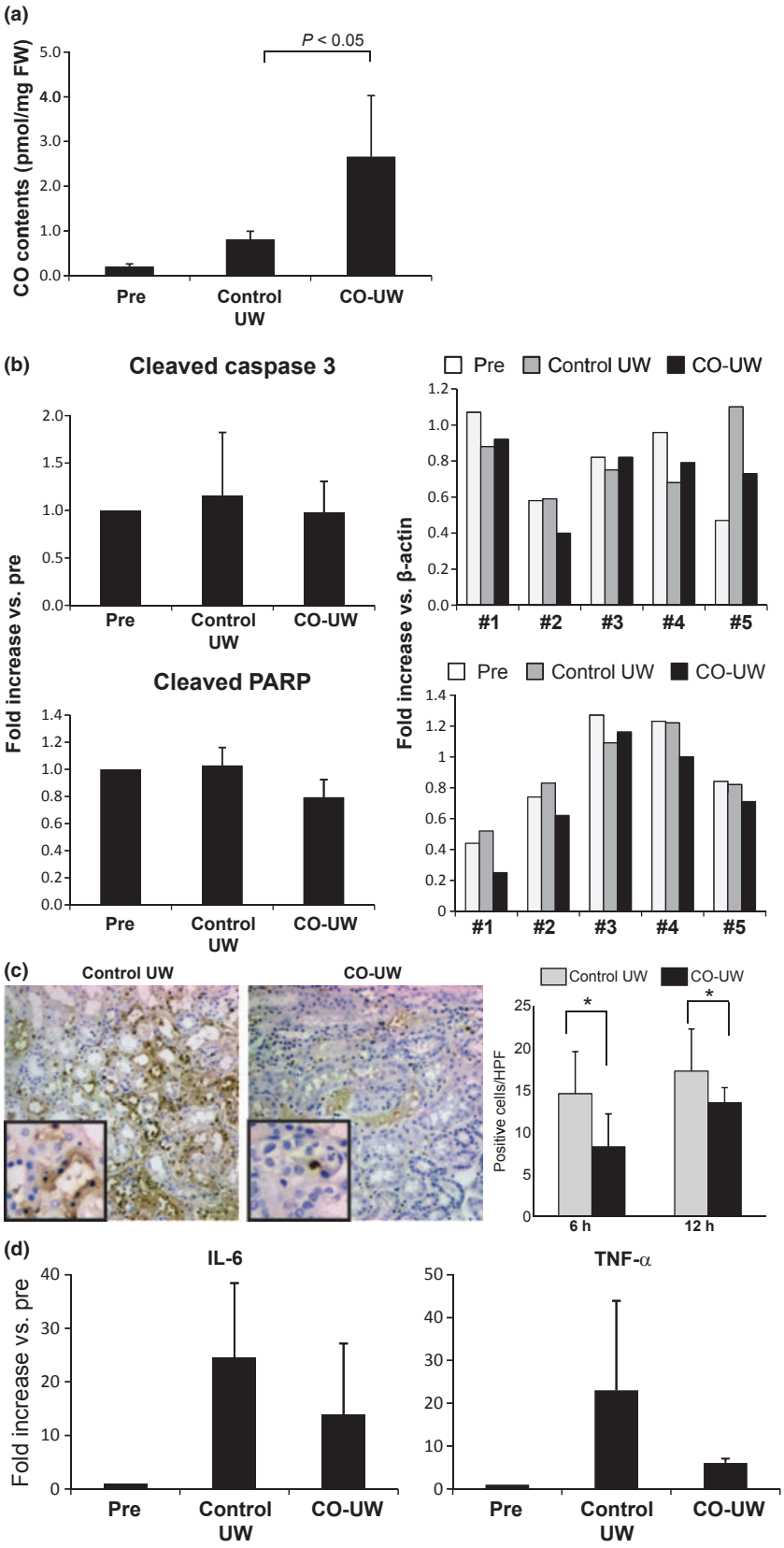

Figure 5 Cold storage (CS) of human kidneys in CO-UW. As soon as nontransplantable human kidney grafts (n = 5) became available, samples were obtained (pre). Upper and lower portions of the kidney grafts were then perfused with control UW (UW) and CO containing UW (CO-UW), and stored with respective solutions for 24 h. (a) Kidney graft samples were obtained at the end of 24 h CS and analyzed for tissue CO contents. (b) Western blot was performed to detect cleaved caspase 3 and cleaved PARP. Portions of kidney grafts after 24 h storage were also used for explant culture for 3–12 h and analyzed for (c) TUNEL analysis (6 and 12 h), and (d) proinflammatory cytokine mRNA levels using RT-PCR at 3 h (TNF-α) and 6 h (IL-6). Representative images of $TUNEL^+$ cells in kidney grafts preserved in control UW or CO-UW for 24 h and cultured for 6 h. Original magnification ×100, insert ×400.

examining effects of carcineurin inhibitors on renal I/R injury show contradictory results depending on the treatment protocols. Although brief administration of carcineurin inhibitors shows protective effects [47–49], prolonged use of these drugs, as well as rapamycin and MMF has been shown to increase renal injury, most likely because of their toxicity to kidneys and interference on repair process [50–52]. CO has been shown to exert protection against nephrotoxic side effects caused by cisplatin [53], and further studies are warranted to determine if CO can protect kidney grafts under immunosuppression.

In summary, the study demonstrates that tissue damage in DCD kidney grafts was substantially augmented during CS. CO in UW solution inhibited mitochondrial damage and caspase pathway activation during CS and resulted in significant amelioration of inflammatory responses and apoptosis after transplantation. Renal graft function and animal survival were significantly improved when kidney grafts were exposed to CO during CS. CO in UW may improve the quality of ECD kidney grafts and reduce the incidence of DGF.

Authorship

KSO, JY, RS, FGL, APS and NM: participated in research design. KSO, JY, RS, FGL, APS and NM: participated in the writing of the paper. KSO, JY, SU, GLP, NG, RS, LYL and NM: participated in the performance of the research. KSO and NM: contributed new reagents or analytic tools. KSO, JY, SU, GLP, NG, LYL and NM: participated in data analysis.

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